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# Defective glomerular [$^3$H]lysoPAF metabolism in the autologous phase of rabbit nephrotoxic nephritis

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**Defective glomerular [$^3$H]lysoPAF metabolism in the autologous phase of rabbit nephrotoxic nephritis.** Glomerular infiltration of blood-derived mononuclear cells contributes to the glomerular injury in the autologous phase of nephrotoxic nephritis (NTN). LysoPAF has recently been shown to be chemotactic for human monocytes, thus its accumulation might account for monocyte recruitment. We investigated [$^3$H]lysoPAF metabolism in isolated glomeruli from normal and NTN rabbits studied both in the heterologous and in the autologous phases of the disease. [$^3$H]lysoPAF was converted to [$^3$H]1-O-alkyl-glycerol and [$^3$H]1-O-alkyl-2-acyl-GPC by phospholipase C and acyltransferase, respectively, both in normal and NTN glomeruli. Glomerular metabolism of [$^3$H]lysoPAF was normal during the heterologous phase of NTN. By contrast, in isolated glomeruli from NTN rabbits studied in the autologous phase of the disease, a significantly lower [$^3$H]lysoPAF degradation occurred with respect to normal ones. This defective degradation resulted in a significantly reduced formation of [$^3$H]1-O-alkyl-glycerol. The apparent $K_m$ for enzymatic conversion of [$^3$H]lysoPAF to [$^3$H]1-O-alkyl-glycerol, determined at 15 minutes as a function of [$^3$H]lysoPAF concentration, was doubled in glomeruli from rabbits studied in the autologous phase of NTN as compared to normal ones, while $V_{max}$ values were similar in the two groups. These results show a defective glomerular lysoPAF degradation in the autologous phase of NTN, likely due to a decreased affinity of phospholipase C to lysoPAF. Altered lysoPAF metabolism results in glomerular accumulation of lysoPAF in the autologous phase of NTN, as shown by significantly higher levels of lysoPAF measured in nephritic glomeruli as compared to normal ones.

Rabbits injected with anti-rabbit glomerular basement membrane (GBM) serum develop a proliferative glomerulonephritis with glomerular fibrin deposition and progressive deterioration of renal function [1]. The disease develops in two subsequent steps: an early (heterologous) phase, occurring within 24 hours after antibody binding to GBM and characterized by complement activation, neutrophil infiltration, proteinuria, and fall in glomerular filtration rate (GFR); and a late (autologous) phase, which ensues at days 7 to 14 after the injection of the antiserum, is dependent on a macrophage/monocyte infiltration and manifests with proteinuria, extracapillary cellular proliferation and renal failure [2–6]. Previous studies in rabbits with nephrotoxic nephritis (NTN) have shown that macrophage depletion abrogates proteinuria and protects from renal failure [5–8], indicating that the recruitment of macrophages in the glomeruli is responsible for the evolution of renal injury in this model. However, the signal(s) for macrophages to accumulate at the glomerular level in such circumstances is still a matter of speculation.

Recent studies on platelet activating factor (PAF), a potent mediator of inflammation released after an immunological challenge from inflammatory cells and platelets [9, 10], may be relevant to this issue. In cell systems PAF is formed by the action of phospholipase $A_2$ that deacylates 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine (alkyl-acyl-GPC) to form 1-O-alkyl-2-lyso-GPC (lysoPAF) which is converted to PAF [11, 12]. PAF may subsequently be inactivated to 1-O-alkyl-acyl-GPC via lysoPAF [13, 14]. Thus lysoPAF is an obligatory intermediate for both biosynthesis and inactivation of PAF. A second metabolic pathway for PAF and lysoPAF has recently been shown, which implies the cleavage of the phosphocholine group by a lysosomal phospholipase C [15, 16] to form the corresponding 1-O-alkyl-glycerols.

Lianos and Zanglis have recently observed that PAF glomerular levels increase in rats with NTN in both phases of the disease [17]. These investigators also provided indirect evidence that glomerular lysoPAF is increased in the autologous phase of NTN [17].

Recently it has been recognized that lysoPAF has its own biological activity consisting in a chemotactic effect for human monocytes in a dose-dependent fashion, a property that may not be shared by PAF [18]. It is possible that lysoPAF accumulates into the glomerulus during the autologous phase of NTN and functions as a stimulus for macrophage/monocyte recruitment within the glomeruli.

The current study was therefore designed with the following aims: (1) to clarify the glomerular metabolism of lysoPAF in the rabbit; (2) to evaluate whether the autologous phase of NTN may be associated with changes in glomerular lysoPAF metabolism, and (3) to evaluate whether lysoPAF accumulates in nephritic glomeruli.

## Methods

### *Materials*

[$^3$H]PAF ([1′,2′-$^3$H]1-0-hexadecyl-2-acetyl-GPC; 56.7 Ci/mmol), [$^3$H]lysoPAF ([1′,2′-$^3$H]1-O-alkyl-2-lyso-GPC; 52.0 Ci/

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mmol), [$^3$H]1-O-alkyl-2-acyl-GPC ([1',2'-$^3$H]1-O-alkyl-2-arachidonyl-GPC; 40 Ci/mmol) were purchased from New England Nuclear (Boston, Massachusetts, USA). [$^3$H]lysoPAF and [$^3$H]1-O-alkyl-2-acyl-GPC were a mixture of isomers with hexadecyl, octadecyl and tetradecyl alkyl moiety. As indicated by New England Nuclear, the distribution of radiolabel among various alkylglyceryl-ethers in [$^3$H]lysoPAF is: hexadecyl- 68%; octadecyl- 10%; tetradecyl- 6%; others 16%. Unlabeled 1-O-hexadecyl-glycerol was from Fluka Chemie (Buchs, Switzerland). Unlabeled 1-O-hexadecyl-2-lyso-GPC (lysoPAF) was from Bachem Feinchemikalien (Bubendorf, Switzerland). 1-O-alkyl-2,3-diacetyl-glycerol was prepared from 1-O-hexadecyl-glycerol by treatment with acetic anhydride as previously described [19]. 1-stearoyl-2-arachidonyl-glycerol was synthesized by hydrolysis with phospholipase C (from Bacillus cereus type II, Boheringer Mannheim, Germany) of L-$\alpha$-phosphatidyl-choline-$\beta$-arachidonyl-$\gamma$-stearoyl (Sigma Chemical Co., St. Louis, Missouri, USA).

HPLC and analytical grade solvents, sodium hydroxide (NaOH) and thin layer chromatography plates, precoated with Silica Gel 60, layer thickness 0.25 mm, were purchased from Merck (Darmstadt, Germany). Silicic acid (100 to 200 mesh) was obtained from Bio-Rad Laboratories (Richmond, California, USA). Bovine serum albumin (BSA fraction V), Dragendorff's reagent, pyridine, 4-N-dimethylamino-pyridine and acetic anhydride were from Sigma.

### *Experimental design*

NTN was induced in male New Zealand white rabbits (Charles River S.p.A., Calco, Italy) weighing 2 to 2.5 kg, by the intravenous injection of 1.5 ml/kg body weight of ram-anti-rabbit GBM serum as previously described [20]. The ram antiserum was given in three administrations at intervals of one hour between the first and the second injection and 40 minutes between the second and the third one. Nephrotoxicity of ram antiserum was assessed by the development of proteinuria, the impairment of renal function and by morphological evaluation of glomeruli with light microscopy, immunofluorescence and electron microscopy as previously described [20].

Three groups of animals were used for the study: (1) normal rabbits ($N$ = 18), (2) rabbits with NTN ($N$ = 8) sacrificed 24 hours following anti-GBM antiserum administration (heterologous phase), (3) rabbits with NTN ($N$ = 18) sacrificed 14 days following anti-GBM antiserum administration (autologous phase).

Rabbits with NTN developed heavy proteinuria (heterologous phase: 6185 ± 3260 mg protein/day, autologous phase: 753 ± 296 mg protein/day as determined by Coomassie blue G, dye-binding assay) [21]. Renal function, measured as creatinine clearance [20], was sharply impaired both 24 hours and 14 days after the antiserum injection in respect to basal values (24 hours: 1.2 ± 0.6 vs. 3.4 ± 0.3 ml/min/kg body wt, $P < 0.01$; 14 days: 0.9 ± 0.4 vs. 3.2 ± 0.5 ml/min/kg body wt, $P < 0.01$). Histological examination of kidney specimens from rabbits sacrificed 24 hours after the antiserum injection revealed glomerular polymorphonuclear cell infiltration and deposition of proteinaceous material in Bowman's space [20]. In kidney specimens from animals sacrificed during the autologous phase of NTN, glomerular infiltrate mainly consisting of mononuclear cells, intra- and extra-capillary fibrin deposition and crescent formation were observed [20].

### *Isolation of glomeruli*

Rabbits were anesthetized by intravenous injection of penthobarbital-sodium. After obtaining specimens for histological examination, kidneys were perfused with saline until they became completely blanched to remove blood-born cells from the glomeruli, then were quickly removed and placed in ice-cold Krebs Ringer phosphate buffer (KRB, 120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$ and 15.6 mM phosphate buffer, pH 7.4). Glomeruli were isolated by differential sieving using a technique previously described [22] which provides about 90% pure glomerular preparation for both normal and NTN kidneys.

### *LysoPAF degradation in normal and NTN glomeruli*

To evaluate [$^3$H]lysoPAF metabolism, isolated glomeruli from normal ($N$ = 4) or nephritic rabbits (heterologous phase, $N$ = 4; autologous phase, $N$ = 4) were centrifuged at 150 × g for five minutes; thereafter the pellet was resuspended in KRB supplemented with 2.5 mM $CaCl_2$ and 0.25% BSA. Aliquots of glomerular suspensions from single animals (2 ml, about 2 mg protein each) were transferred to polypropylene tubes containing [$^3$H]lysoPAF (1.5 $\mu$Ci, about 28.8 pmol, in ethanol) that had been previously dried under a nitrogen stream. Samples were incubated in duplicate for 150 minutes at 37°C under constant shaking.

In additional experiments isolated glomeruli from normal ($N$ = 8) or NTN rabbits (autologous phase, $N$ = 8) were pooled. To assess the time course of lysoPAF metabolism aliquots of the glomerular suspensions (about 1 mg protein) from normal or NTN rabbits were incubated with [$^3$H]lysoPAF (28.8 pmol) for various time periods (5 to 150 min). To evaluate the apparent $K_m$ and $V_{max}$ of the enzymatic degradation of [$^3$H]lysoPAF to [$^3$H]1-O-alkyl-glycerol, glomeruli (about 0.5 mg protein) from normal and NTN rabbits were incubated at 37°C for 15 minutes with increasing amounts (range 0.5 to 25 $\mu$mol/liter) of lysoPAF containing 1 $\mu$Ci of [$^3$H]lysoPAF. Each incubation was performed in triplicate.

All incubations were stopped by adding 4 ml acidified methanol, pH 3. After gentle mixing for 30 minutes, samples were centrifuged at 2000 × g for 15 minutes to precipitate denatured glomerular proteins, then the pellet was resuspended in 2 ml acidified methanol, vortexed and centrifuged once again. Supernatants were extracted according to the Bligh and Dyer procedure [23], and pellets were dissolved in NaOH 1N for protein determination [24]. The chloroform-rich phase of extracts was dried under nitrogen and purified according to the method of Pinckard, Farr and Hanahan [25] with the following modifications: samples were resuspended in 1 ml of chloroform/methanol 9:1 (vol/vol) and loaded on a column packed with 0.5 g of silicic acid (Bio-Rad Laboratories, 100 to 200 mesh) activated overnight at 100°C. The column was eluted sequentially with 15 ml of chloroform, 15 ml of acetone, 15 ml of acetone/methanol (1:1 vol/vol), 30 ml of chloroform/methanol (1:4 vol/vol). The third and the fourth fractions of the column, containing phospholipid metabolites, were combined and dried. An aliquot of each sample was dissolved in 200 $\mu$l of chloroform/methanol 2:1 (vol/vol) and analyzed by HPLC. Samples were chromatographed by isocratic elution on a Perkin Elmer

Silica A Column (2.6 × 250 mm; Perkin-Elmer, Norwalk, Connecticut, USA), connected to a Beckman model 342 Gradient Liquid Chromatograph (Beckman Instruments Inc., Berkeley, California, USA). The eluent was acetonitrile/methanol/85% phosphoric acid 130:5:1.5 (vol/vol/vol) as described by Chen and Kou [26]. The flow rate was 1.5 ml/min and 0.75 ml fractions were collected and assayed for radioactivity by scintillation counting. Labeled products were separated and identified on the basis of the retention time of the corresponding [$^3$H] standards. Portions of the extracts as well as of the various fractions of the silicic acid column were used for calculation of recoveries which were above 90% and 85% for the extraction and the silicic acid column, respectively.

The chloroform fraction of the silicic acid column, containing neutral lipid metabolites, was dried, redissolved in chloroform/methanol 9:1 (vol/vol) and spotted on a Silica-Gel thin-layer plate. Ten micrograms of unlabeled 1-O-alkyl-glycerol were applied closed to the samples. The thin-layer plates were developed in a solvent system of chloroform/methanol 98:3 (vol/vol) (solvent system I). Unlabeled lipid standard was sprayed with a solution of 0.025% of Rhodamine 6G (Merck) in ethanol and visualized under ultraviolet light. The plates were scraped at 1 cm intervals and scraping was placed into plastic vials containing 10 ml of scintillation mixture (Instagel II, Packard Instruments, Downers Grove, Illinois, USA) for determinations of radioactivity using a Beckman LS 1701 liquid scintillation counter (Beckman Instruments).

### *Subcellular fractionation*

In additional experiments, subcellular fractions (membranes, microsomes, cytosol) were prepared from isolated glomeruli from two normal rabbits. Glomeruli were homogenized four times for 30 seconds with an Ultra-Turrax homogenizer (Jankle and Kunkel GMBH and Co., KG, IKA-WERK Staufen, Germany) in 10 ml of KRB, and centrifuged at 500 × g for two minutes in order to eliminate glomerular debris. The supernatant was centrifuged at 10,000 × g for 15 minutes and the pellet (including plasma membranes, lysosomes and mithocondria) saved for further purification. The 10,000 × g supernatant was centrifuged at 100,000 × g for 60 minutes to prepare microsomes (100,000 × g pellet) and cytosol (100,000 × g supernatant). The 10,000 × g pellet was resuspended in KRB, homogenized twice for 30 seconds and then recentrifuged at 100,000 × g for 60 minutes to get rid of any trapped cytosol. Homogenization and all subsequent procedures were carried out at 0 to 4°C. Membranes, microsomes and cytosol were divided into three aliquots and incubated for 15 minutes at 37°C in 2 ml of KRB supplemented with 0.25% BSA and 2.5 mM $CaCl_2$ in the presence of 5 nmol (2.5 μM) [$^3$H]lysoPAF. At the end of the incubation period samples were extracted and processed as described above.

### *LysoPAF degradation in lung homogenate*

To evaluate whether possible alterations of lysoPAF metabolism in NTN rabbits were confined to the kidney, the kinetic parameters of [$^3$H]lysoPAF degradation to [$^3$H]1-O-alkyl-glycerol in the lung homogenate were also evaluated. Lungs from normal ($N$ = 3) and NTN rabbits (autologous phase, $N$ = 3) were removed, finely minced with a razor blade and homogenized in 20 ml of ice-cold KRB using an Ultra-Turrax homogenizer (3 × 30 seconds). The homogenate was centrifuged at 25,000 × g for 10 minutes at 4°C, thereafter the pellet was washed with 20 ml of ice-cold KRB and centrifuged once again. The pellet was resuspended in KRB supplemented with 0.25% BSA and 2.5 mM $CaCl_2$ and aliquots (about 2 mg protein each) were incubated at 37°C for 15 minutes with increasing amounts (range 0.5 to 25 μmol/liter) of lysoPAF containing 1 μCi of [$^3$H]lysoPAF. Each incubation was performed in duplicate. Samples were extracted and analyzed as described above.

### *Identification of products of [$^3$H]lysoPAF metabolism*

The labeled metabolite comigrating with 1-O-alkyl-glycerol on TLC was extracted with dichloromethane. A portion of the extract was subjected to alkaline hydrolysis (NaOH 2 N) for 30 minutes at room temperature. The product was analyzed by TLC (solvent system I) and $R_F$ values were compared with those of authentic 1-O-alkyl-glycerol. Another portion was subjected to acetylation according to the procedure described by Benfenati et al [19]. In brief, the extract was dissolved in 200 μl of acetic anhydride and heated at 80°C for three hours. The product was analyzed by TLC using a solvent system of chloroform/methanol/glacial acetic acid 96:4:1 (vol/vol) (solvent system II) and $R_F$ values were compared with those of synthetic 1-O-alkyl-2,3-diacetyl glycerol. Labeled phospholipid metabolites coeluting with standard [$^3$H]1-O-alkyl-2-acyl-GPC and [$^3$H]lysoPAF in HPLC were also analyzed by TLC using a solvent system of chloroform/methanol/water 65:35:4 (vol/vol) (solvent system III). Authentic standards of L-α-phosphatidylcholine-β-arachidonyl-γ-stearoyl and lysoPAF were co-chromatographed and visualized by Dragendorff's reagent. A portion of the labeled compound coeluting with standard [$^3$H]1-O-alkyl-2-acyl-GPC was subjected to brief alkaline hydrolysis (NaOH 2 N) for 30 minutes at room temperature. The product was analyzed by TLC (solvent system III) and $R_F$ values were compared with those of lysoPAF and L-α-phosphatidylcholine-β-arachidonyl-γ-stearoyl standard. Another portion was subjected to phospholipase C treatment (500 U/ml at 37°C for 90 min) [27]. The product was analyzed by TLC (solvent system I) and $R_F$ values were compared with those of standard 1-O-stearoyl-2-arachidonyl-glycerol.

### *LysoPAF levels in normal and NTN glomeruli*

Glomerular lysoPAF content was indirectly evaluated by radioimmunoassay (RIA) after its chemical conversion to PAF. Isolated glomeruli from normal ($N$ = 4) or nephritic rabbits (heterologous phase, $N$ = 4; autologous phase, $N$ = 6) were resuspended in ice-cold KRB. Glomerular suspensions from each animal were divided into two aliquots which were spiked with approximately 5,000 cpm of [$^3$H]PAF standard in order to calculate recoveries. Lipids were subsequently extracted with acidified methanol followed by the Bligh and Dyer procedure as described above. Both isolation of glomeruli and lipid extraction were performed at 0°C to avoid degradation of glomerular phospholipids. One of the two aliquots of each glomerular lipid extract was subsequently dissolved in 300 μl of chloroform/pyridine 3:1 (vol/vol) and acetylated in the presence of acetic anhydride, 30 μl, and of 4-N-dimethylamino-pyridine, 2 mg, for 18 hours at room temperature as described [17]. This procedure completely acetylates glomerular lysoPAF to PAF. Acetylated and not acetylated glomerular lipid extracts were subsequently

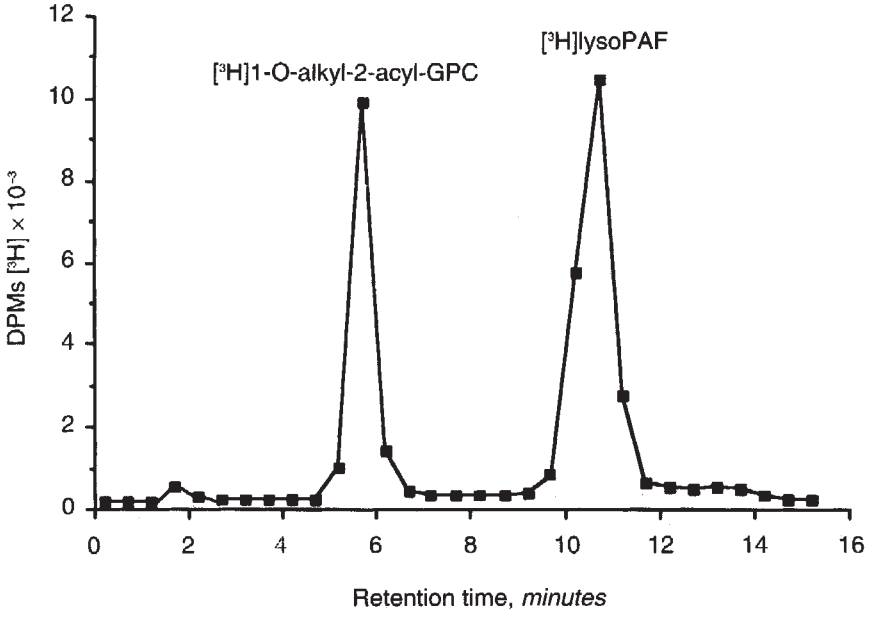


**Fig. 1.** *HPLC radiochromatogram of normal glomeruli phospholipid extract from 150 minutes of incubation at 37°C with [³H]lysoPAF (28.8 pmol).* The glomerular extract was purified and analyzed by straight phase HPLC as described in **Methods**. Solvent system, acetonitrile: methanol: phosphoric acid (130:5:1.5 vol/vol/vol), flow rate 1.5 ml/minutes.

purified by a silicic acid column as described above. Purified samples were assessed for PAF content by a specific radioimmunoassay as previously described [28]. Glomerular lysoPAF levels were calculated by subtracting data from not-acetylated aliquots (which reflected PAF levels) to total glomerular PAF (PAF plus lysoPAF) measured in acetylated samples. Results were expressed as pmol/mg glomerular protein.

### *Statistical analysis*

All results are expressed as mean ± SD. Results were analyzed by the Kruskall-Wallis test and Ryan's procedure or by one-way or two-way analysis of variance with Tukey-Cicchetti test for multiple comparisons, as appropriate. Probability values $<0.05$ were considered to be statistically significant. Linear regression analysis was performed to evaluate the "apparent" $K_m$ and $V_{max}$ of the enzymatic degradation of [³H]lysoPAF to [³H]1-O-alkyl-glycerol in normal and NTN tissues. Ninety-five percent Confidence Interval of the difference between slopes was calculated.

## Results

Figure 1 shows a representative fractionation of radiolabeled phospholipid extract from normal glomeruli incubated for 150 minutes with [³H]lysoPAF. HPLC analysis yielded a radiochromatogram containing two major peaks coeluting with authentic [³H]1-O-alkyl-2-acyl-GPC and [³H]lysoPAF, respectively. The TLC analysis of neutral lipid metabolites (Fig. 2) showed a single band with $R_F = 0.23$ which comigrated with authentic 1-O-alkyl-glycerol. After 150 minutes incubation, the percentage of radioactivity comigrating with [³H]1-O-alkyl-glycerol and [³H]1-O-alkyl-2-acyl-GPC was 59.7 ± 9.1 and 15.7 ± 4.9%, respectively ($N = 4$). These data suggest that in isolated glomeruli from normal rabbits [³H]lysoPAF metabolism occurs by the action of either a phospholipase C or an acyltransferase.

An alternative mechanism to account for the observed formation of 1-O-alkyl-glycerol from lysoPAF would involve the stepwise actions of microsomal lysophospholipase D and phosphatidate phosphohydrolase [29]. To investigate such a possibility, additional experiments were performed, where subcellular fractions from normal glomeruli were incubated with

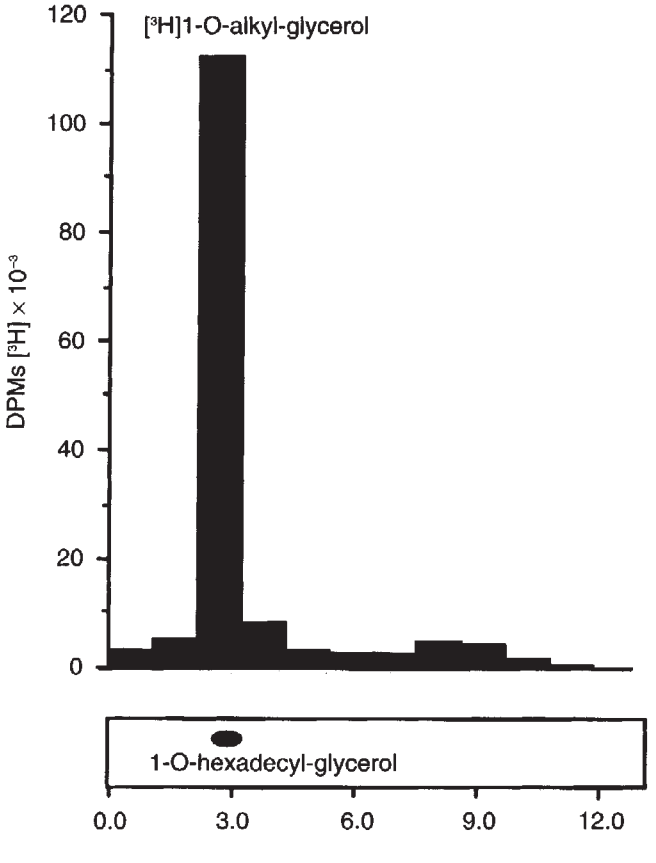


**Fig. 2.** *TLC of neutral lipid metabolites from isolated normal rabbit glomeruli incubated with [³H]lysoPAF (28.8 pmol) for 150 minutes at 37°C.* Solvent system, chloroform: methanol 98:3 (vol/vol).

**Table 1.** Subcellular localization of [$^3$H]1-O-alkyl-glycerol synthesis in glomeruli from normal rabbits

| | Membranes | Microsomes | Cytosol |
|---|---|---|---|
| % Of total activity | 63.4 ± 6.06 | 23.3 ± 2.07 | 13.3 ± 1.5 |

Subcellular fractions from normal glomeruli were prepared by sequential centrifugation and incubated with [$^3$H]lysoPAF for 15 min at 37°C. Lipids were extracted and analyzed by TLC. Results are expressed as % of total enzymatic activity. Values are means ± SD ($N$ = 3).

[$^3$H]lysoPAF and then extracted and purified for [$^3$H]1-O-alkyl-glycerol determination. As shown in Table 1, 63% of total enzymatic activity, expressed as ng alkyl-glycerol/minute/mg glomerular protein, were located in the membrane fraction, whereas little activity was found both in the microsomes and in the cytosol.

We further investigated lysoPAF metabolism in isolated glomeruli from NTN rabbits studied both in the heterologous and in the autologous phase of the disease. After 150 minutes of incubation, the HPLC and TLC profiles of [$^3$H]lysoPAF metabolites in NTN glomeruli were qualitatively similar to control ones. However, in isolated glomeruli from animals sacrificed during the autologous phase of NTN, formation of [$^3$H]1-O-alkyl-glycerol was significantly reduced than in normal glomeruli and in glomeruli from animals sacrificed during the heterologous phase of the disease ($P < 0.05$ vs. normal and NTN 24 hours). On the contrary, the formation of [$^3$H]1-O-alkyl-2-acyl-GPC was similar in normal and NTN glomeruli. Results, expressed as pmol/mg glomerular protein, are depicted in Figure 3.

The time course of [$^3$H]lysoPAF degradation by isolated glomeruli from both normal and NTN rabbits studied in the autologous phase of the disease is illustrated in Figure 4. In isolated glomeruli from normal rabbits [$^3$H]lysoPAF was degraded greatly and time-dependently until 150 minute incubation. The decrease of [$^3$H]lysoPAF content was accompanied by an increase in either [$^3$H]1-O-alkyl-glycerol or [$^3$H]1-O-alkyl-2-acyl-GPC levels. [$^3$H]1-O-alkyl-glycerol was the major metabolite at each incubation time considered. When [$^3$H]lysoPAF was incubated with isolated glomeruli from NTN rabbits, its metabolism was reduced as compared to normal glomeruli. Results, expressed as residual pmol [$^3$H]lysoPAF, showed a significant difference ($P < 0.05$) between the two groups at incubation time of 30 to 150 minutes (Fig. 4). The defective degradation of [$^3$H]lysoPAF in NTN glomeruli resulted in a significantly ($P < 0.01$) reduced formation of [$^3$H]1-O-alkyl-glycerol at incubation time of 15 to 150 minutes. On the contrary, the levels of the second metabolite [$^3$H]1-O-alkyl-2-acyl-GPC were comparable in normal and NTN glomeruli.

Conversion of [$^3$H]lysoPAF to [$^3$H]1-O-alkyl-glycerol in isolated glomeruli from normal and NTN rabbits (autologous phase) was further studied as a function of the substrate concentration. The "apparent" $K_m$ and $V_{max}$ values for [$^3$H]lysoPAF conversion to [$^3$H]1-O-alkyl-glycerol, determined in 15-minute incubation experiments, were deduced from Lineweaver-Burk plots of 1/V versus 1/[lysoPAF] (Fig. 5). The maximum velocity for hydrolysis of [$^3$H]lysoPAF was similar in NTN and in normal glomeruli (0.17 vs. 0.16 nmol/min/mg glomerular protein), whereas "apparent" $K_m$ of the enzymatic

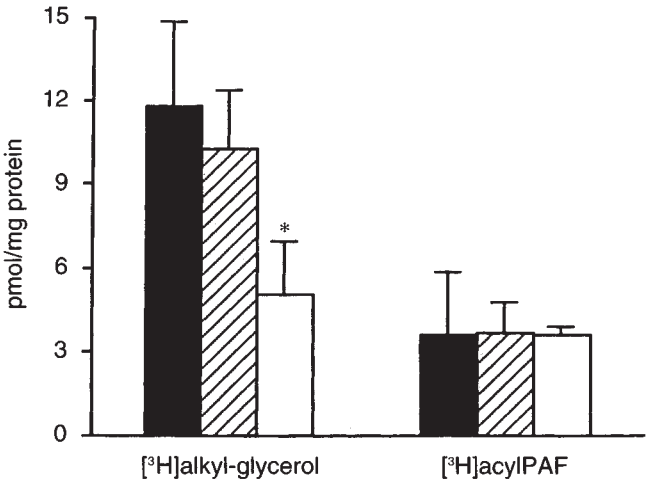


**Fig. 3.** *[$^3$H]lysoPAF metabolism in isolated glomeruli from normal (■) and NTN (heterologous phase, NTN 24 hours, ▨; autologous phase, NTN 14 days, □) rabbits.* Isolated glomeruli from single animals (about 2 mg protein) were incubated with [$^3$H]lysoPAF (28.8 pmol) at 37°C for 150 minutes. Each incubation was performed in duplicate. Samples were purified and analyzed as described in **Methods**. Results are expressed as pmol/mg glomerular protein. Values are means ± SD ($N$ = 4). * $P < 0.05$ vs. normal and NTN (24 hours). [$^3$H]acylPAF, [$^3$H]alkyl-2-acyl-GPC.

reaction was doubled in NTN glomeruli as compared to normal ones (18.13 vs. 8.66 μM). The 95% Confidence Interval of the difference between slopes of the Lineweaver-Burk plots of normal and NTN glomeruli ranged from −55.5 to −36.3, indicating that the two slopes were significantly different. By contrast, the "apparent" $V_{max}$ and $K_m$ for [$^3$H]lysoPAF conversion to [$^3$H]1-O-alkyl-glycerol in lung homogenate from NTN rabbits (autologous phase) were quite comparable to normals ($V_{max}$: normals 0.057 ± 0.009, NTN 0.06 ± 0.02 nmol/min/mg protein; $K_m$: normals 19.12 ± 10.6, NTN 23.20 ± 4.71 μM, $N$ = 3).

To evaluate whether lysoPAF accumulates within the kidney in the autologous phase of NTN, glomerular lysoPAF levels were evaluated in normal and nephritic rabbits. As shown in Figure 6, significantly higher lysoPAF content was measured in glomeruli from rabbits sacrificed during the autologous phase of NTN in comparison to normal glomeruli (255 ± 119.7 vs. 113 ± 11.5 pmol/mg protein, $P < 0.05$). By contrast, glomerular lysoPAF in NTN rabbits studied in the heterologous phase of the disease was comparable to normal animals (135 ± 26.5 pmol/mg protein).

The identification experiments performed on products obtained from incubation of glomeruli and lung homogenate with [$^3$H]lysoPAF revealed the following: when the labeled product comigrating with 1-O-alkyl-glycerol was subjected to acetylation, it comigrated with 1-O-alkyl-2,3-diacetyl glycerol standard in solvent system II, indicating that the polar head group of [$^3$H]lysoPAF had been cleaved. The same compound was resistant to alkaline hydrolysis; this was suggestive of the presence of an ether bond in position sn-1. When the compound comigrating with [$^3$H]1-O-alkyl-2-acyl-GPC standard was subjected to mild alkaline hydrolysis, it was quantitatively converted to a product comigrating with lysoPAF on TLC. This

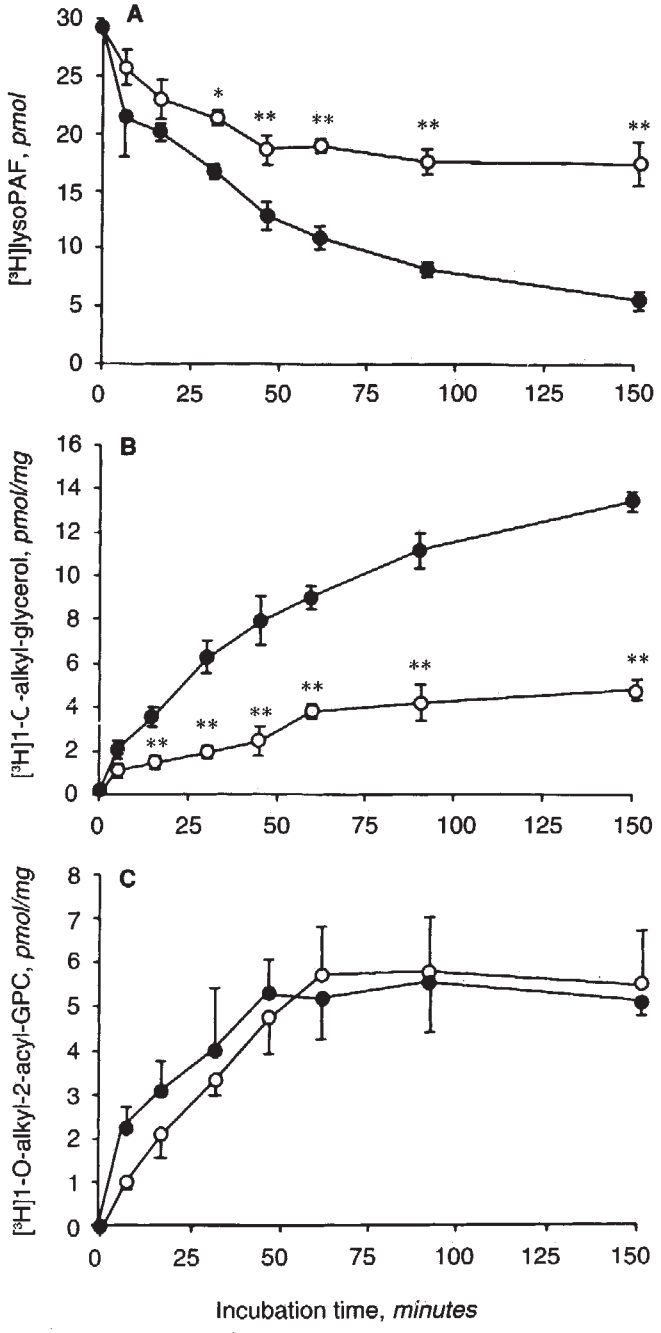


**Fig. 4.** *Time course of [3H]lysoPAF metabolism by isolated glomeruli from normal (●) and NTN (14 days, ○) rabbits.* Glomeruli (about 1 mg/protein) were incubated with [3H]lysoPAF (28.8 pmol) at 37°C for various time points (range: 5 to 150 min, $N = 3$ for each point). Glomerular suspensions were subsequently extracted and analyzed as described in **Methods**. Results are expressed as residual pmol for [3H]lysoPAF (**A**) and as pmol/mg glomerular protein for metabolic products (**B** and **C**). Values are means ± SD. * $P < 0.05$, ** $P < 0.01$ vs. normal.

was indicative that the compound was esterified in position sn-2 and had an ether bond in position sn-1. Finally, when the same product was subjected to phospholipase C treatment it comigrated with 1-stearoyl-2-arachidonyl-glycerol standard in solvent system I.

## Discussion

With the present study we have documented that normal rabbit glomeruli rapidly and extensively metabolize lysoPAF to 1-O-alkyl-glycerol and to 1-O-alkyl-2-acyl-GPC.

Previous studies in rabbit platelets [30] and in rabbit alveolar macrophages [31] have shown that lysoPAF is converted to 1-O-alkyl-2-acyl-GPC by the enzyme acyltransferase. Moreover, conversion of lysoPAF and PAF to 1-O-alkyl-glycerol by specific lysosomal phospholipase C has been recently documented in isolated cells [16] and in tissues [15]. The conversion of lysoPAF to 1-O-alkyl-glycerol has been also attributed to the stepwise actions of microsomal lysophospholipase D and phosphatidate phosphohydrolase [29]. In our experiments with subcellular fractions we found that as much as 63% of the total 1-O-alkyl-glycerol from rabbit glomeruli is accounted for by the membrane fraction whereas 23% was recovered in the microsomal fraction. These data would indicate that in rabbit glomeruli lysoPAF conversion to 1-O-alkyl-glycerol occurs mainly via a membrane linked phospholipase C and secondary via the actions of lysophospholipase D and phosphatidate phosphohydrolase.

Here we have also documented that lysoPAF metabolism is markedly reduced in isolated glomeruli from NTN rabbits studied during the autologous phase of the disease, as compared to normal rabbit glomeruli. The reduced lysoPAF degradation was mostly due to its reduced conversion to 1-O-alkyl-glycerol. This abnormality is likely a consequence of a defective phospholipase C activity, which mediates the main metabolic pathway of lysoPAF in rabbit glomeruli. By contrast glomerular acyltransferase activity was comparable in NTN and normal glomeruli as indicated by findings of similar amount of [3H]1-O-alkyl-2-acyl-GPC after exposure to radiolabeled lysoPAF. At variance with the autologous phase, glomerular lysoPAF metabolism was normal in the heterologous phase of rabbit NTN. It is possible that infiltrating leukocytes contribute to lysoPAF degradation within the glomerulus in the heterologous phase of NTN thus leading to a normal "net" lysoPAF metabolism.

To clarify whether the defective phospholipase C activity in nephritic glomeruli was the consequence of a reduced affinity of the enzyme for the substrate or rather due to a lower absolute amount of the enzyme, we calculated the $K_m$ and $V_{max}$ of [3H]lysoPAF degradation to 1-O-alkyl-glycerol. Finding of similar $V_{max}$ in normal and nephritic glomeruli but increased $K_m$ in the latter, suggests that the abnormality might be due to a reduced affinity of phospholipase C for lysoPAF. It is possible that the affinity of phospholipase C for lysoPAF is reduced by virtue of an endogenous inhibitor which may bind to the active site of the enzyme. Identical $V_{max}$ of the enzymatic degradation of lysoPAF but increased $K_m$ in NTN glomeruli would indeed be consistent with a competitive inhibition. Alternatively, inflammatory cells present in NTN glomeruli could act as "lysoPAF-sucklers" and make lysoPAF less available to phospholipase C.

$V_{max}$ and $K_m$ of [3H]lysoPAF conversion to [3H]1-O-alkyl-glycerol was comparable in lung homogenate from normal and

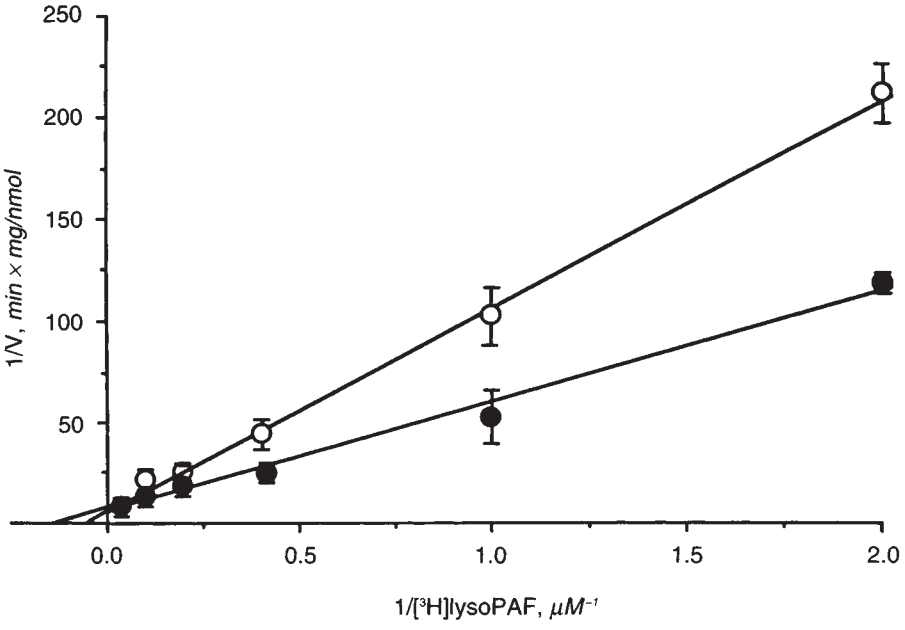


**Fig. 5.** *Double-reciprocal plot of [$^3$H]alkyl-glycerol formation by isolated glomeruli from normal (●) and NTN (14 days, ○) rabbits as a function of concentration of [$^3$H]lysoPAF.* Isolated glomeruli (about 0.5 mg protein) were incubated with different concentrations of [$^3$H]lysoPAF (range 0.5 to 25 μmol/liter, $N = 3$ for each point) for 15 minutes and subsequently purified and analyzed as described in Methods. Values are means ± SD.

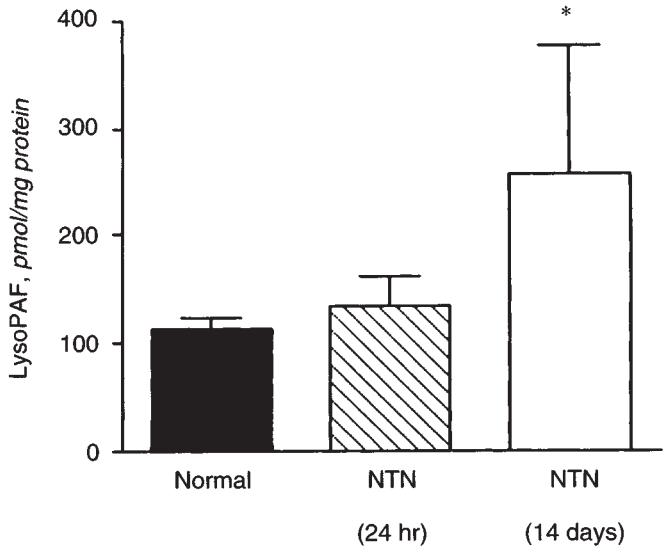


**Fig. 6.** *LysoPAF content in isolated glomeruli from normal (N = 4) and NTN (heterologous phase: NTN 24 hours, N = 4; autologous phase: NTN 14 days, N = 6) rabbits.* Results are expressed as pmol/mg glomerular protein. Values are means ± SD. * $P < 0.05$ vs. normal.

nephritic rabbits, suggesting that the altered lysoPAF metabolism in the autologous phase of NTN was confined to the kidney.

Several cell types in the glomerulus might account for the altered metabolism of lysoPAF in the autologous phase of NTN. Mesangial cells [32, 33] as well as vascular endothelial cells [34] are capable of degrading lysoPAF. Furthermore, macrophages, which accumulate in the nephritic glomeruli, can convert lysoPAF to 1-O-alkyl-2-acyl-GPC [31]. However, failure to find increased formation of [$^3$H]1-O-alkyl-2-acyl-GPC in NTN glomeruli makes unlikely the possibility that macrophages play a crucial role in the glomerular metabolism of lysoPAF in the autologous phase of rabbit NTN.

Defective glomerular lysoPAF metabolism in the autologous phase of NTN would result in a local accumulation of lysoPAF in the glomerulus. The possibility that lysoPAF is accumulating in glomeruli of NTN animals is supported by our finding that lysoPAF levels, measured as PAF after chemical acetylation of glomerular extracts, were increased in rabbits with NTN studied in the autologous phase of the disease. These data are in agreement with those previously reported by Lianos and Zanglis [17] in nephritic rats. Efforts in finding the possible mediator(s) of the glomerular macrophage accumulation in NTN are of great relevance since the degree of macrophage infiltration may determine the subsequent evolution of the disease to renal failure [5, 6, 8]. LysoPAF, unlike PAF, is a potent chemotactic agent for human monocytes/macrophages as shown in a recent study by Quinn, Parthasarathy and Steinberg [18]. Actually, these investigators found that lysoPAF exerts a dose-dependent chemotactic activity to human monocytes while PAF was inactive. Thus, glomerular lysoPAF accumulation during the autologous phase of rabbit NTN may be a signal for macrophages to migrate within the glomerulus. This possibility is strengthened by finding that in the heterologous phase of NTN, which is associated with normal glomerular lysoPAF metabolism, macrophage accumulation does not occur [35].

In conclusion, our present findings indicate that (a) normal rabbit glomeruli metabolize lysoPAF to 1-O-alkyl-glycerol and to 1-O-alkyl-2-acyl-GPC that account respectively for 60% and 16% of lysoPAF metabolism; (b) the glomerular conversion of lysoPAF to 1-O-alkyl-glycerol is reduced in the autologous phase of rabbit NTN resulting in a less efficient lysoPAF degradation; (c) such a defect in lysoPAF degradation is likely the consequence of a lower affinity of the enzyme phospholipase C for lysoPAF; and (d) reduced lysoPAF degradation is associated to accumulation of lysoPAF within the nephritic glomerulus.

Further studies are needed to identify the mechanism(s) of the reduced phospholipase C activity in NTN glomeruli and to evaluate whether such a defect is the signal for macrophage accumulation during the autologous phase of NTN.

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